Modular TRAPP complexes regulate intracellular protein trafficking through multiple Ypt/Rab GTPases in *S. cerevisiae*

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Abstract

Ypt/Rab are key regulators of intra-cellular trafficking in all eukaryotic cells. In yeast, Ypt1 is essential for endoplasmic reticulum (ER)-to-Golgi transport, whereas Ypt31/32 regulate Golgi-to-plasma membrane and endosome-to-Golgi transport. TRAPP is a multi-subunit complex that acts as an activator of Ypt/Rab GTPases. Trs85 and Trs130 are two subunits specific for TRAPP III and TRAPP II, respectively. Whereas TRAPP III was shown to act as a Ypt1 activator, it is still controversial whether TRAPP II acts as a Ypt1 or Ypt31/32 activator. Here, we use GFP-Snc1 as a tool to study transport in Ypt and TRAPP mutant cells. First, we show that expression of GFP-Snc1 in trs85∆ mutant cells results in temperature sensitivity. Second, we suggest that in ypt1ts and trs85∆, but not in ypt31∆/32ts and trs130ts, mutant cells, GFP-Snc1 accumulates in the ER. Third, we show that over-expression of Ypt1, but not Ypt31/32, can suppress both the growth and GFP-Snc1 accumulation phenotypes of trs85∆ mutant cells. In contrast, over-expression of Ypt31, but not Ypt1, suppresses the growth and GFP-Snc1 transport phenotypes of trs130ts mutant cells. These results provide genetic support for functional grouping of Ypt1 with Trs85-containing TRAPP III and Ypt31/32 with Trs130-containing TRAPP II.
Introduction

Transport of membranes and proteins through the endocytic and exocytic pathways connects membrane-bound intracellular compartments with the plasma membrane (PM) and the cell milieu. The endoplasmic reticulum (ER) is the compartment into which membrane and cargo proteins are translocated en route to all the other cellular compartments. From the ER, membrane and proteins are transported through the Golgi apparatus to the PM.

Ypt/Rab GTPases regulate trafficking between cellular compartments [1-3]. In yeast, Ypt1 is required for ER-to-Golgi transport [4, 5], whereas the Ypt31/32 functional pair plays a role at the trans-Golgi in both Golgi-to-PM and endosome-to-Golgi transport [6, 7]. In addition, Ypt1 and Ypt31/32 play a role in autophagy [8-10]. Autophagy is a cellular process induced by stress in which cytosolic and membrane proteins are engulfed by a double-membrane organelle termed autophagosome to be delivered to the lysosome for degradation [11, 12].

Ypt/Rabs are activated by guanine-nucleotide exchange factors (GEFs) and the GEF for Ypt1 and Ypt31/32 is the TRAPP complex. TRAPP is a multi-subunit modular complex [13], which exists in at least three forms. TRAPP I, which contains five essential subunits, acts as a GEF for Ypt1 [14, 15], and is required for ER-to-Golgi transport [16]. Trs85, a TRAPP subunit non-essential for cell viability, plays a role in autophagy [17, 18]. A Trs85-containing TRAPP complex, termed TRAPP III, also acts as a Ypt1 GEF [9]. TRAPP II, which contains Trs120 and Trs130 in addition to TRAPP I subunits, functions at
the trans-Golgi [19]. However, currently the Ypt GEF specificity of the TRAPP II complex is under dispute.

Based on biochemical and genetic evidence we have proposed that TRAPP I activates Ypt1 in ER-to-Golgi transport, whereas Ypt31/32 act at the trans Golgi in both Gogli-to-PM and endosome-to-Golgi transport, and TRAPP II acts as their GEF at least in the former step [6, 14, 20]. This model is currently being challenged claiming that Ypt1 functions in endosome-to-Golgi transport in addition to its role in ER-to-Golgi transport [21], and that TRAPP II acts as a GEF for Ypt1 in both steps [22-24]. Here we study the physiological relationship between Ypt1 and Ypt31/32 and two TRAPP complexes, TRAPP III and TRAPP II. Data presented in this study provides support to the idea that TRAPP II acts with Ypt31/32, and not with Ypt1, in endosome-to-Golgi transport.

GFP-Snc1 has been extensively used as a PM recycling marker in yeast [6, 21, 25, 26]. Snc1 is a v-SNARE that plays a role in fusion of trans-Golgi vesicles with the PM. For multiple rounds of vesicle fusion, Snc1 recycles back from the PM to the Golgi via endosomes [25]. We have previously used GFP-Snc1 to reveal a role for Ypt31/32 in endosome-to-Golgi transport [6]. Recently, a role for Ypt1, together with TRAPP II, has been proposed in the recycling of GFP-Snc1 from the PM using YPT1 alleles defective specifically in Snc1-GFP trafficking [21]. In addition, a role for Trs85 in endosome-to-Golgi transport was also suggested based on intra-cellular accumulation of Snc1-GFP in trs85Δ mutant cells [26]. In contrast, our results suggest that in ypt1ts and trs85Δts mutant cells GFP-Snc1 accumulates in the ER, supporting a role for Ypt1 and
Trs85 in exit of membrane proteins from the ER. Because accumulation of GFP-Snc1 in the ER of trs85Δts mutant cells is coupled with a cell-growth defect, we propose that accumulation of proteins in the ER can cause cell toxicity. Moreover, over-expression analyses support functional grouping of Ypt31/32 with the Trs130-containing TRAPP II complex in endosome-to-Golgi transport and of Ypt1 with the Trs85-containing TRAPP complex in ER exit of over-expressed membrane proteins. It is not clear whether this role of Trs85 is related to its established function in autophagy.
Results

Biochemical analysis has shown that TRAPP I acts as a Ypt1 GEF [14, 15]. However, the substrate for TRAPP II complex is currently controversial [20, 23]. Recently, the Trs85-containing TRAPP III complex was suggested to act as a GEF for Ypt1 and not Ypt32 [9]. However, in the published experiment there was no positive control for a Ypt32 GEF and it did not include Ypt31. Here, we add biochemical support to the idea that TRAPP III acts as a GEF for Ypt1, but not Ypt31/32, by including the missing positive control. The Ypt GEF activity of the Trs85-containing TRAPP complex purified from yeast lysates was compared to that of the Bet5-containing TRAPP complexes, using a GDP-release assay. Because the Bet5 subunit is common to the three known TRAPP complexes, purified GST-Bet5 TRAPP contains all the TRAPP complexes including TRAPP II. Under our reaction conditions, in addition to acting as a Ypt1 GEF, GST-Bet5 purified TRAPP acts as a GEF for Ypt31 and Ypt32. In contrast, the GST-Trs85 purified complex acts as a GEF only for Ypt1 (Figure S1). Thus, the Trs85-containing TRAPP, like TRAPP I, acts as a GEF for Ypt1, but not for Ypt31/32.

To study the physiological relationship between Ypt1 and Ypt31/32 and two TRAPP complexes, TRAPP II and TRAPP III, we followed the transport of GFP-Snc1 in mutants defective in Ypt and TRAPP function.

**GFP-Snc1 accumulates in the ER of ypt1ts and trs85Δts mutant cells.**
The functional pair of Snc1 and Snc2 acts as a v-SNARE that cycles between the Golgi, PM and endosomes [27]. GFP-Snc1 has been used commonly as a PM recycling marker in yeast [25]. In wild type cells, GFP-Snc1 localizes mostly to the PM, whereas in mutant cells defective in PM recycling it accumulates in endosomes. Using this marker, we have previously shown that Ypt31/32 play a role in endosome-to-Golgi transport [6]. A role for several TRAPP subunits in this step, including the two TRAPP II-specific subunits, Trs120 and Trs130 [28], and the three non-essential TRAPP subunit, Trs33, Trs65 and Trs85 [26], was also suggested based on accumulation of intracellular GFP-Snc1. Recently, a role for Ypt1 in endosome-to-Golgi transport was suggested [21], and \( \text{trs85}\Delta \) was identified in a screen for genes required for endosome-to-Golgi transport [26]. Even though a role for Trs85 and Ypt1 in autophagy has been established [9, 10], they could also play a role in endosome-to-Golgi transport, individually or together. For example, Ypt31/32 and Trs130 function both in exit from the trans-Golgi and in endosome-to-Golgi transport [6, 7, 19, 28]. Therefore, we re-examined whether Ypt1 and Trs85 function in the recycling of GFP-Snc1 from the PM.

GFP-Snc1 was integrated into the \( URA3 \) locus in wt, \( ypt1ts \) and \( \text{trs85}\Delta \) mutant cells (these cells express both Snc1 and Snc2) [25]. GFP-Snc1 expressing \( \text{trs85}\Delta \), but not \( \text{trs65}\Delta \), mutant cells exhibit a temperature-sensitive growth phenotype (Figure 1A). The linkage of the temperature-sensitive growth phenotype with over-expression of GFP-Snc1 in \( \text{trs85}\Delta \) mutant cells was verified by co-segregation of the temperature sensitivity with integrated GFP-Snc1 and
trs85Δ, and by over-expression of GFP-Snc1 from a 2µ plasmid (Figure S2). Thus, over-expression of GFP-Snc1 in trs85Δ mutant cells confers a temperature-sensitive phenotype. We termed the mutant strain in which GFP-Snc1 is integrated and TRS85 is deleted trs85Δts. This mutant was used as a tool in microscopic analysis, to study GFP-Snc1 transport phenotype, and in over-expression analysis, to determine genetic interactions between the Ypts and TRAPP subunits.

The accumulation of GFP-Snc1 in ypt1ts and trs85Δts mutant cells at their permissive (26°C) and non-permissive (37°C) temperatures was determined using live-cell microscopy (Figure S3). In both ypt1ts and trs85Δts mutant cells GFP-Snc1 accumulates internally at 37°C. This internal GFP-Snc1 accumulation was compared to the accumulation in ypt31Δ/32ts and trs130ts mutant cells, in which GFP-Snc1 accumulates in endosomes already at the permissive temperature (under conditions that allow Golgi-to-PM transport, [6]). However, whereas in ypt31Δ/32ts and trs130ts mutant cells GFP-Snc1 accumulates in puncta, the internal accumulation in ypt1ts and trs85Δts mutant cells appears as rings. Because Ypt1 functions in ER-to-Golgi transport and in yeast ER appears as rings around the nucleus [29, 30], we wished to determine whether the GFP-Snc1 rings in ypt1ts and trs85Δts mutant cells are around the nucleus.

To determine the cellular compartment in which GFP-Snc1 accumulates in ypt1ts and trs85Δts mutant cells, it was co-localized with nuclear and endosomal markers. The nucleus was visualized by Htb1-CFP (Histone H2B, [31]). In both ypt1ts and trs85Δts mutant cells GFP-Snc1 accumulates in rings around the
nucleus labeled with Htb1-CFP. In contrast, in \textit{ypt31Δ/32ts} and \textit{trs130ts} mutant cells the puncta are not associated with the nucleus (Figure 1B-C).

Localization of GFP-Snc1 to endosomes was determined by staining cells with the fluorescent dye FM4-64 for 5 minutes. As expected for a protein that recycles through endosomes, there is some localization of GFP-Snc1 to endosomes in wild-type cells. In \textit{ypt1ts} and \textit{trs85Δts} mutant cells the limited co-localization of GFP-Snc1 with the endosomal staining is similar to that seen in wild-type cells. In contrast, under the same conditions (as previously reported, [6, 28]), in \textit{ypt31Δ/32ts} and \textit{trs130ts} mutant cells intracellular GFP-Snc1 localizes mostly to endosomes (Figure 2).

Together, the co-localization analysis of GFP-Snc1 with nuclear and endosomal markers suggest that in \textit{ypt1ts} and \textit{trs85Δts}, but not in \textit{ypt31Δ/32ts} and \textit{trs130ts}, mutant cells Snc1 accumulates in the ER.

**The non-recycling marker, GFP-Snc1-PEM, accumulates in the ER of \textit{ypt1ts} and \textit{trs85Δ} mutant cells.**

To further characterize the GFP-Snc1 localization defect in \textit{ypt1ts} and \textit{trs85Δts} mutant cells, we used the GFP-Snc1-PEM protein. This hybrid protein contains the Sso1 trans-membrane domain and two point mutations, V40A and M43A, which interfere with endocytosis and cause accumulation on the PM [25]. Therefore, GFP-Snc1-PEM does not accumulate in endosomes in mutant cells defective in PM recycling [6, 32, 33]. Importantly, this protein can be used as a
probe for defects in the biosynthetic transport of GFP-Snc1 because it is not endocytosed.

In wild-type cells, GFP-Snc1-PEM accumulates on the PM both at 26°C and 37°C. As expected, in ypt31Δ/32ts and trs130ts mutant cells at 26°C, GFP-Snc1-PEM localizes mostly to the PM (Figure 3A). This localization supports the idea that in these mutant cells intra-cellular accumulation of GFP-Snc1 is caused by a PM recycling defect. At 37°C, GFP-Snc1-PEM accumulates in both ypt31Δ/32ts and trs130ts mutant cells (Figure 3A). This intra-cellular accumulation of GFP-Snc1-PEM indicates a block in transport of this protein to the PM. Because at 37°C, ypt31Δ/32ts and trs130ts mutant cells are defective in Golgi-to-PM transport [7, 34], GFP-Snc1-PEM probably accumulates in the Golgi.

In ypt1ts and trs85Δ mutant cells at 26°C, GFP-Snc1-PEM localizes to the PM and, especially in ypt1ts mutant cells, it also accumulates inside the cells. At 37°C, while there is GFP-Snc1-PEM on the PM, it also localizes to internal rings (Figure 3A-B). Moreover, like the GFP-Snc1 rings that accumulate in these mutant cells at 37°C, the GFP-Snc1-PEM rings are also surrounding the nucleus (Figure 3C). Finally, like over-expression of GFP-Snc1, over-expression of GFP-Snc1-PEM in trs85Δ, but not trs65Δ, mutant cells confers a cell growth defect (Figure 1A). Thus, we suggest that GFP-Snc1-PEM, like GFP-Snc1, accumulates in the ER of ypt1ts and trs85Δ mutant cells.

These results suggest that whereas in ypt31Δ/32ts and trs130ts mutant cells GFP-Snc1 and GFP-Snc1-PEM accumulate in endosomes and Golgi,
respectively, in ypt1ts and trs85Δ mutant cells both GFP-Snc1 and GFP-Snc1-PEM accumulate in the ER en route to the PM.

**Over-expression of Ypts in TRAPP mutants**

Recently, the observation that ypt1 mutant cells, like TRAPP II-specific subunit mutants, accumulate internalized GFP-Snc1 was used to support the claim that TRAPP II acts as a GEF for Ypt1 to regulate endosome-to-Golgi transport [21]. To test this idea, we performed an over-expression analysis. The effect of over-expression of Ypt1 or Ypt31/32 on the following two phenotypes of trs85Δts and trs130ts mutant cells was determined: internal accumulation of GFP-Snc1 and cell growth.

Ypt1 and Ypt31 were over-expressed from 2µ plasmids. Over-expression of Ypt1 specifically suppresses the growth defect of ypt1ts, but not ypt31Δ/32ts, mutant cells. Conversely, over-expression of Ypt31 suppresses the growth defect of ypt31Δ/32ts, but not ypt1ts, mutant cells (Figure S4).

If Ypt1 plays a role in GFP-Snc1 recycling from the PM together with the Trs130-containing TRAPP II complex, it was expected that over-expression of Ypt1 would suppress the GFP-Snc1 recycling defect of trs130ts mutant cells. However, we found that Ypt31/32, but not Ypt1, can suppress this defect in ypt31Δ/32ts and trs130ts mutant cells (Figure 4C-E and Figure S5B-C). In contrast, Ypt1, but not Ypt31, can suppress the intracellular accumulation of GFP-Snc1 in ypt1ts and trs85Δts mutant cells (Figure 4A-B and 4E). Ypt32 also cannot suppress the intracellular accumulation of GFP-Snc1 in ypt1ts and
trs85Δts mutant cells (data not shown). These results argue against the idea that Ypt1 plays a role with Trs130-containing TRAPP II in endosome-to-Golgi transport, and further support the physiological relationship between Ypt31/32 and the Trs130-containing TRAPP II complex.

If the growth defect of the trs85Δts mutant cells is caused by ER accumulation of GFP-Snc1, we expected that suppression of the GFP-Snc1 ER accumulation by over-expression of Ypt1 should also suppress the temperature growth defect of these cells. Moreover, if Ypt31/32, and not Ypt1, function together with TRAPP II, we expect that over-expression of Ypt31/32, but not Ypt1, would suppress the growth defect of trs130ts mutant cell. Indeed, over-expression of Ypt1, but not Ypt31, can suppress the temperature-sensitive growth phenotype of ypt1ts and trs85Δts mutant cells (Figure 5A and Figure S4A). Over-expression of Ypt32 also cannot suppress the temperature-sensitive growth phenotype of ypt1ts and trs85Δts mutant cells (data not shown). In contrast, the temperature-sensitive growth phenotype of ypt31Δ/32ts and trs130ts mutant cells can be suppressed by over-expression of Ypt31 or Ypt32, but not Ypt1 (Figure 5B and Figures S4B and S5A).

In summary, genetic analysis presented here reveals that while over-expression of Ypt1 can suppress the two GFP-Snc1-induced phenotypes of trs85Δts mutant cells, GFP-Snc1 accumulation in the ER and temperature sensitivity, it does not suppress those of trs130ts mutant cells. Importantly, the two GFP-Snc1-induced phenotypes of trs130ts mutant cells, internal accumulation of GFP-Snc1 and temperature sensitivity, can be suppressed by
over-expression of Ypt31/32. Therefore, these results provide genetic support to grouping Ypt1 with Trs85-containing TRAPP III and Ypt31/32 with Trs130-containing TRAPP II (Figure 5C).
Discussion

Here, the v-SNARE GFP-Snc1 is used as a transport marker to support two conclusions. First, GFP-Snc1 and GFP-Snc1-PEM accumulate in the ER of \textit{ypt1ts} and \textit{trs85}\Delta mutant cells en route to the PM. Second, over-expression analysis supports physiological grouping of Ypt1 with the Trs85-containing TRAPP III complex and of Ypt31/32 with the Trs130-containing TRAPP II complex (Figure 5C). The importance of each conclusion is discussed below.

Using GFP-Snc1 as a cargo marker

Using traditional exocytic markers, like invertase and CPY, it has been established that \textit{ypt1ts} mutant cells exhibit an ER transport block [4], whereas \textit{ypt31\Delta/32ts} and \textit{trs130ts} exhibit a Golgi block [7, 19, 28]. Using CPY (Prc1) as a marker, it was recently concluded that \textit{trs85}\Delta mutant cells do not exhibit an ER block for this luminal vacuolar protease (even though a weak kinetic block is observed, [9]).

Here, we show that the membrane-associated GFP-Snc1 can also be used as an exocytic cargo marker. In wild-type cells GFP-Snc1 is inserted into the ER membrane like all other proteins that contain a trans-membrane domain. From the ER, the v-SNARE Snc1 is transported to the Golgi where it plays a role in Golgi-to-PM transport. Snc1 can then be recycled back to the Golgi through endosomes for multiple rounds of vesicular transport [25]. GFP-Snc1 has been used as a PM-recycling marker to show that \textit{ypt31\Delta/32ts} and \textit{trs130ts} mutant cells are defective in endosome-to-Golgi transport [6, 28]. We show here that
GFP-Snc1 can also be used as an exocytic cargo marker in mutants defective in exit from the ER. Whereas in ypt31Δ/32ts and trs130ts it accumulates in endosomes or the Golgi, in ypt1ts and trs85ts it accumulates in the ER. The apparent discrepancy between transport of CPY [9] and GFP-Snc1 (here) in trs85Δ mutant cells might be due to the difference between the two markers: CPY is an endogenous luminal protein, whereas GFP-Snc1 is a tagged over-expressed membrane protein.

**Ypt1 and Trs85 act in ER exit**

Accumulation of internalized GFP-Snc1 in trs85Δ and ypt1 mutant strains has been previously reported. It was taken as an indication that Ypt1 and Trs85 play a role in endosome-to-Golgi transport [21, 26]. Here we show that in ypt1ts and trs85Δts mutant cells GFP-Snc1 accumulates in the ER and not during PM recycling. This idea is supported by three observations: First, GFP-Snc1 accumulates in rings around the nucleus which do not overlap with internalized FM4-64, suggesting ER localization. Second, similar accumulation of the non-recycling GFP-Snc1-PEM protein, indicates that this accumulation is not caused by a PM recycling defect, but, rather by a transport defect. Third, the observation that over-expression of Ypt1 cannot suppress the endosomal accumulation of GFP-Snc1 in trs130ts mutant cells, but can suppress the ER accumulation of GFP-Snc1 in trs85Δts mutant cells, supports the idea that Ypt1 does not have a role in TRAPP II-mediated endosome-to-Golgi transport. Therefore, we can conclude that Trs85 does not play a role in endosome-to-Golgi transport. As for
Ypt1, while \textit{ypt1ts} mutant cells do not exhibit a block in this transport step, the idea that specific \textit{YPT1} alleles are defective in endosome-to-Golgi transport [21] is not addressed here, because these alleles were not used in this study. However, data presented here indicate that even if Ypt1 plays a role in endosome-to-Golgi transport, it does not act together with TRAPP II in that step (see below).

Why does GFP-Snc1 accumulate in the ER of \textit{ypt1ts} and \textit{trs85\Delta ts} mutant cells? There are two possible explanations for this phenotype. Because Ypt1 is required for ER-to-Golgi transport and \textit{ypt1ts} mutant cells exhibit a block in this step at their restrictive temperature [4], it is possible that GFP-Snc1 is blocked in the ER of \textit{ypt1ts} and \textit{trs85\Delta ts} mutant cells en route to the Golgi. In this scenario, at high temperatures, Trs85 plays a role in ER-to-Golgi transport. Alternatively, because both Ypt1 and Trs85 play a role in autophagy [9, 10], it is possible that in \textit{ypt1ts} and \textit{trs85\Delta ts} mutant cells excess GFP-Snc1 fails to be shuttled through the autophagy pathway for degradation in the lysosome/vacuole. Future studies should address this question.

Deletion of \textit{TRS85} results in temperature sensitivity only if GFP-Snc1 protein is over-expressed in this mutant strain. The correlation shown here between ER accumulation of GFP-Snc1 and GFP-Snc1-PEM at 37°C and a temperature sensitive growth phenotype of \textit{trs85\Delta} mutant cells expressing these tagged proteins, suggests that accumulation of proteins in the ER can cause cell toxicity. Such toxicity can be related to the role of Trs85 and Ypt1 in autophagy.
Alternatively, it can be caused by an ER-to-Golgi transport block due to depletion of exocytic machinery by the ER-accumulated GFP-Snc1.

**Grouping Ypt1 with Trs85 and Ypt31/32 with Trs130**

We have previously shown that Ypt1 and Ypt31/32 are essential for Golgi entry and exit, respectively [4, 5, 7]. We have also shown that TRAPP I and TRAPP II act as GEF for Ypt1 and Ypt31/32, respectively [14, 20]. This idea is supported by biochemical and genetic evidence [20, 35, 36], and agrees with the assignment of TRAPP I and TRAPP II to the cis- and trans-Golgi, respectively, in the exocytic pathway [16, 19]. However, this view has been challenged by negative biochemical results [23, 24, 37], and a role for Ypt1 with TRAPP II in endosome-to-Golgi transport was suggested [21]. Data presented here further support a physiological role of Ypt31/32, and not Ypt1, with TRAPP II also in endosome-to-Golgi transport. While it is possible that GFP-Snc1 accumulates in endosomes in addition to its accumulation in the ER of ypt1 mutant cells not used in the current study, over-expression analysis presented here argues against the possibility that it acts with TRAPP II in this transport step. Thus, our genetic analysis further supports grouping of Ypt31/32, but not Ypt1, with TRAPP II in endosome-to-Golgi transport.

We propose that the modular TRAPP complexes act as GEFs for multiple Ypts, and not just for Ypt1. Currently, there is a dispute regarding the Ypt specificity of the different TRAPP complexes. Results presented here agree with previously published data that Trs85-containing TRAPP III acts as a GEF for
Ypt1 [9]. Importantly, we show here that TRAPP III does not act as a Ypt31/32 GEF under conditions in which GST-Bet5-purified TRAPP has such an activity (Figure S1). Our over-expression analysis provides genetic support to the idea that Ypt1 acts together with Trs85 whereas Ypt31/32 act together with Trs130 (Figure 5C).

In summary, studies presented here further support our view of a role for TRAPP I and Trs85-containing TRAPP III with Ypt1 in exit from the ER and a role for TRAPP II with Ypt31/32 in exit from the trans-Golgi and in the recycling of PM proteins to the Golgi.
Materials and Methods

Strains, Plasmids, and Reagents

Strains and plasmids used in this study are summarized in the Supplementary Information, Table S1. For genetic interaction experiments, Ypt1, Ypt31 and Ypt32 in 2µ plasmids and their corresponding empty plasmids (ϕ, pRB684, pRS425 and yep351, respectively) were used. All yeast transformations were done using the lithium acetate method [38]. *E. coli* transformation was done using electroporation.

For live-cell microscopy observation, GFP-Snc1 was integrated into NSY340 (ypt31Δ/32ts) and NSY128 (wild type) with pRS406 GS GFP-Snc1 (pNS568, [25]) to create YLY132 and YLY130, respectively. TRS85 was deleted from the wild type strain (YLY130) with KAN<sup>R</sup> cassette to create trs85Δts (YLY1347). YLY130 was used to mate with NSY1082 (ypt1ts), NSY991 (TRS130-HA), and NSY992 (trs130ts). The diploids were sporulated and tetrads were dissected to obtain the following GFP-Snc1 tagged mutants and wild type strains: YLY1665 (ypt1ts) and YLY1664 (YPT1), YLY1771 (trs130ts) and YLY1770 (TRS130). GFP-Snc1-PEM was integrated into ypt3Δ/32ts, ypt1ts, trs130ts and their corresponding wild-type strains with pRS406 GSSOM GFP-Snc1-PEM (pNS571, [25]) to create YLY1613 (ypt1ts-GFP-Snc1-PEM); YLY1583 (ypt31Δ/32ts-GFP-Snc1-PEM); YLY1585 (trs130ts-GFP-Snc-PEM) and their corresponding wild-type strains. TRS85 was deleted from YLY1582 to obtain YLY1651 (trs85Δ-GFP-Snc-PEM). Htb1-CFP was integrated into the above GFP-Snc1 tagged strains using pYL227 (a gift from B. Schwappach, Georg-
August-Universität Göttingen, Germany) for marking the nucleus.

Antibodies used in this study are: rabbit anti-glucose-6-phosphate dehydrogenase (G6PDH, Sigma-Aldrich, MO, USA); affinity-purified rabbit anti-Ypt31 [7]; affinity-purified rabbit anti-Ypt1 [5]; horseradish peroxidase linked anti-rabbit and anti-mouse IgG (Amersham Biosciences, Little Chalfont, UK).

All chemical reagents were purchased from Amersco (Fair Lawn, NJ), unless otherwise noted. Other reagents used in this study: SynaptoRed, also known as FM4-64 (Molecular Probes, Eugene, OR), DAPI (Roche Diagnostics, Indianapolis, IN), Geneticin (Gibco Laboratories, Grand Island, NY), Restriction enzymes and buffers (Takara Biotechnology, Dalian, China).

**Yeast Culture Conditions**

For genetic interaction, cells were grown overnight at 26°C in YPD or minimal (SC) media, normalized to the same density and spotted onto agar plates in ten-fold serial dilutions. Plates were incubated at different temperatures for genetic interaction assays. For live-cell fluorescence microscopy, yeast cultures were grown at permissive temperature (26°C) in rich (YPD) or selective (when plasmid is used) media to log phase, and switched to a restrictive temperature (37°C) for 1.5 hours. Cells were either directly observed, or subjected to DAPI staining or FM4-64 staining as described below.

**Fluorescence Microscopy**

Direct fluorescence microscopy of temperature-sensitive yeast cells was performed as described in [6] to localize Snc1, Snc1-PEM and Htb1. DAPI staining was performed according to protocol suggested by the manufacturer.
FM4-64 staining for endosomes (5 minutes) was done as previously described [21]. Slides were visualized using Nikon inverted research microscope Eclipse Ti (Tokyo, Japan). More than five fields were collected for each sample. Each experiment was repeated at least twice.

**Cell Lysates and Immuno-blot Analysis**

For checking the expression level of Ypt1 or Ypt31 in cells used for the genetic interaction assay, five OD$_{600}$ of overnight cell cultures were lysed in 100 µl of Laemmli buffer supplemented with equal volume of glass beads (BioSpec Products, Bartlesville, OK) and vortexed for 2 minutes. The supernatant of a 13,000 rpm spin (2 minutes) was subjected to immuno-blot analysis with anti-Ypt1 or anti-Ypt31 antibodies, together with anti-G6PDH for serving as a loading control.

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Figure Legends

Figure 1. GFP-Snc1 accumulates in the rings around the nuclei of \textit{ypt1ts} and \textit{trs85\textDelta ts} mutant cells. A. \textit{trs85\Delta}, but not \textit{trs65\Delta}, mutant cells expressing GFP-Snc1 or GFP-Snc1-PEM exhibit a temperature-sensitive growth phenotype at 37°C. The \textit{TRS85} or \textit{TRS65} gene was deleted in the following three strains: wild type (BY4741, left), chromosomally-tagged GFP-Snc1-PEM (YLY1582, middle) and chromosomally-tagged GFP-Snc1 (YLY130, right). Cells were grown on YPD plates at 26°C (top) and 37°C (bottom). Four ten-fold serial dilutions are shown from top to bottom. B. GFP-Snc1 accumulates in the ER of \textit{ypt1ts} and \textit{trs85\Delta ts}, but not of \textit{ypt31\Delta/32ts} and \textit{trs130ts}, mutant cells. Wild-type and mutant cells expressing chromosomally-tagged GFP-Snc1 and Htb1-CFP, as a nuclear marker, were grown in YPD medium at 26°C to mid-log phase and then shifted to 37°C for 1.5 hours. GFP-Snc1 and Htb1-CFP were visualized using live-cell fluorescence microscopy. In \textit{ypt1ts} and \textit{trs85\Delta ts}, but not in \textit{ypt31\Delta/32ts} or \textit{trs130ts}, GFP-Snc1 accumulates around nuclei, which is indicative of ER localization. Bar, 7 µm. Arrows point to GFP-Snc1 on the PM and arrowheads to perinuclear GFP-Snc1 circle. C. Quantification of data presented in panel B: shown is percent of cells with an internal GFP-Snc1 ring around the nucleus, which is marked with Htb1-CFP. At least 200 cells were counted in at least two fields for each strain. Error bars represent STDEV.

Figure 2. GFP-Snc1 accumulates in endosomes of \textit{ypt31\Delta/32ts} and \textit{trs130ts}, but not of \textit{ypt1ts} and \textit{trs85\Delta ts}, mutant cells. A. Wild-type and
mutant cells expressing chromosomally-tagged GFP-Snc1 were stained with FM4-64 for 5 minutes to label early endosomes and were visualized using live-cell fluorescence microscopy. Cells grown in YPD medium at 26°C to mid-log phase, were shifted to 37°C for 85 minutes, stained with FM4-64 for 5 minutes and kept on ice until visualization. Bar, 7 µm. Arrowheads point to GFP-Snc1 that localizes to early endosomes marked with FM4-64. B. Quantification of data presented in panel A: shown is percent of cells with internal GFP-Snc1 that does not overlap with FM4-64. At least 200 cells were counted in at least three fields for each strain. Error bars represent STDEV.

Figure 3. GFP-Snc1-PEM accumulates in the rings around the nuclei of ypt1ts and trs85Δts mutant cells. A. Like GFP-Snc1, GFP-Snc1-PEM accumulates in ring-like structures in ypt1ts and trs85Δ, but not in ypt31Δ/32ts or trs130ts mutant cells. Wild type and mutant yeast cells expressing chromosomally-tagged GFP-Snc1-PEM were used for live-cell fluorescence microscopy as described in Figure S3 legend. GFP-Snc1-PEM accumulates inside ypt1ts and trs85Δ mutant cells under the same conditions that cause intracellular accumulation of GFP-Snc1; namely 26°C and 37°C for ypt1ts and 37°C for trs85Δ. In contrast, GFP-Snc1-PEM does not accumulate inside ypt31Δ/32ts or trs130ts mutant cells at 26°C, under conditions that GFP-Snc1 does accumulate (see Figure S3). DIC images (on each side) show the contour of cells; Bar, 7 µm. Arrowheads point to internal GFP-Snc1-PEM. B. Quantification of data presented in panel A: shown is percent of cells with an
internal GFP-Snc1-PEM ring. At least 200 cells were counted in at least three fields for each strain; error bars represent STDEV. C. The GFP-Snc1-PEM rings in ypt1ts and trs85Δ mutant cells are around the nuclei. Ypt1ts and trs85Δ mutant cells were grown at 26°C to mid-log phase and shifted to 37°C for 1.5 hours. The cells were fixed in ethanol, stained with DAPI to mark nuclei and visualized by fluorescence microscopy. Arrowheads point to internalized GFP-Snc1-PEM. The GFP-Snc1-PEM ring-like structures accumulating in these mutant cells are around nuclei indicating ER staining. Bars, 7 μm. Right: *% cells indicates percent cells with an internal GFP-Snc1-PEM ring around the nucleus, which is stained with DAPI; ± represents STDEV.

Figure 4. Ypt1 suppresses the GFP-Snc1 transport phenotype of trs85Δts, whereas Ypt31 suppresses the phenotype of trs130ts. The localization of chromosomally-tagged GFP-Snc1 was determined by live-cell fluorescence microscopy in wild type and mutant cells transformed with 2μ plasmids: Ypt1, and Ypt31, and their corresponding empty plasmids (ϕ:pRB684 and pRS425, respectively). Cells were grown in selective medium (to keep the plasmids) to mid-log phase at 26°C and then shifted to 37°C for 1.5 hours. Plasmids are shown at the top of panel A; Bar, 7 μm. Wild type cells transformed with the different plasmids show the same GFP-Snc1 PM localization; shown on the left are wild type cells transformed with empty vector (ϕ,pRB684). All mutants accumulate internalized GFP-Snc1 (compare the left panels for wild type and mutant with empty plasmid (ϕ)). Over-expression of Ypt1, but not Ypt31,
suppresses the GFP-Snc1 phenotype of \textit{ypt1ts} (A) and \textit{trs85\Delta ts} (B) mutant cells. In contrast, over-expression of Ypt31, but not Ypt1, suppresses the GFP-Snc1 phenotype of \textit{ypt31\Delta/32ts} (C) and \textit{trs130ts} (D). In the GFP-Snc1 panels, arrows point to GFP-Snc1 on the PM in wild type and suppressed mutant cells; arrowheads point to intracellular GFP-Snc1 seen in mutant cells.

\textbf{E.} Quantification of data presented in panels A-D. Shown is percent of cells with GFP-Snc1 on the PM. At least 200 cells were counted in at least four fields for each strain. Error bars represent STDEV.

\textbf{Figure 5.} Ypt1 suppresses the growth defect of \textit{trs85\Delta ts}, whereas Ypt31 suppresses the growth defect of \textit{trs130ts}. Mutant cells, \textit{trs85\Delta ts} (A), and \textit{trs130ts} (B), and their corresponding wild-type cells, all expressing chromosomally tagged GFP-Snc1, were transformed with the following 2\(\mu\) plasmids: Ypt1, and Ypt31, and their corresponding empty plasmids (\(\phi : \text{pRB684 and pRS425, respectively}\)). Cell growth on plates was tested at 26°C and 37°C; four ten-fold serial dilutions are shown from top to bottom. Over-expression of Ypt1, but not Ypt31, suppresses the growth defect of \textit{trs85\Delta ts} (A). In contrast, over-expression of Ypt31, but not Ypt1, suppresses the growth defect of \textit{trs130ts} (B). Over-expression of Ypt1 and Ypt31 in the transformants was verified by immunoblot analysis using anti-Ypt1 and anti-Ypt31 antibodies, respectively; G6PDH indicates equal loading (shown at the bottom of each panel).

\textbf{C.} Summary of the over-expression analysis described here: Specific suppression of \textit{trs85\Delta ts} and \textit{trs130ts} mutant cells by Ypt1 and Ypt31/32,
respectively, supports physiological grouping of Ypt1 with Trs85-containing TRAPP III and Ypt31/32 with Trs130-containing TRAPP II complex.
References

17. Meiling-Wesse, K., U.D. Epple, R. Krick, H. Barth, A. Appelles, C. Voss, E.L. Eskelinen, and M. Thumm, *Trs85 (Gsg1), a component of the TRAPP complexes,


Supplementary Information

Table S1

Figures S1-S5

Supplementary Materials and Methods
Figure 2

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Figure 5

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Ypt1  Ypt31  G6PDH

B. WT  trs130ts

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Ypt1  Ypt31  G6PDH

C.

Ypt/Rab GTPase:
- Ypt1
- Ypt31/32

TRAPP subunit:
- Trs85
- Trs130

TRAPP complex:
- III
- II